

AMENDMENTS

Amendments to the Specification

1. Please replace the paragraph 48 with the one below:

Another embodiment of the present invention provides a modified neurotoxin comprising a botulinum toxin (such as a botulinum toxin type A) which includes a structural modification which is effective to alter a biological persistence of the modified neurotoxin relative to an identical neurotoxin without the structural modification. The structural modification can comprise a deletion of amino acids 416 to 437 from a light chain of the neurotoxin ~~(Fig. 3)~~ of SEQ ID NO: 29.

2. Please replace the paragraph 49 with the one below:

In still another embodiment of the present invention there is provided a modified neurotoxin (such as a botulinum toxin type A) which includes a structural modification which is effective to alter a biological persistence of the modified neurotoxin relative to an identical neurotoxin without the structural modification. The structural modification can comprise a deletion of amino acids 1 to 8 from a light chain of the neurotoxin ~~(Fig. 3)~~ of SEQ ID NO: 29.

3. Please replace the paragraph 50 with the one below:

Still further in accordance with the present invention there is provided a modified neurotoxin, such as a botulinum toxin type A, which includes a structural modification which is effective to alter a biological persistence of the modified neurotoxin relative to an identical neurotoxin without the structural modification. The structural modification may comprise, for example, a deletion of 2 or more amino acids from 1 to 20 and a deletion of 2 or more amino acids from 398 to 437 from a light chain of the neurotoxin of SEQ ID NO: 29. In one embodiment, the structural modification comprises a deletion of amino acids 1 to 8 and 416 to 437 from a light chain of the neurotoxin ~~(Fig. 3)~~ of SEQ ID

NO: 29. In some embodiments, the structural modification comprises a deletion of amino acids 1 to 9 and 416 to 437 from a light chain of the neurotoxin of SEQ ID NO: 29. With regard to deletion on either the 1-8 or 1-9 amino acids; after synthesis the initial Methionine (M) of, for example, BoNT/A is apparently posttranslationally removed within Clostridia. Amino acids 1 – 8 do not include the initial Met residue. If one includes the initial Met residue, then amino acids 1 – 9 are removed. Of course a recombinant toxin would need a Met residue incorporated to start protein synthesis. It may or may not be removed following synthesis.

4. Please replace the paragraph 51 with the one below:

For example, a native synthesized BoNT/A can comprise: MPFVNKQFNYKD (SEQ ID NO: 14), whereas a native processed BoNT/A can comprise PFVNKQFNYKD (SEQ ID NO: 15). Thus a proposed 8 amino acid deletion would retain the YKD amino acid residues, while a recombinantly produced deletion would retain the MYKD amino acid residues.

5. Please replace the paragraph 52 with the one below:

Still further in accordance with the present invention, there is provided a modified botulinum toxin, such as a modified botulinum toxin type A, which includes a structural modification effective to alter a biological persistence of the modified neurotoxin relative to an identical neurotoxin without said structural modification. The structural modification can comprise a substitution of leucine at position 427 for an alanine and a substitution of leucine at position 428 for an alanine in a light chain of said neurotoxin ~~(Fig. 3)~~ of SEQ ID NO: 29.

6. Please replace the paragraph 72 with the one below:

Fig. 1 shows localization of GFP-botulinum toxin A light chain in (nerve growth factor) NGF-differentiated live PC12 cells visualized on a fluorescence inverted microscope.

The arrow indicates that GFP-botulinum toxin A light chain localizes to the plasma membrane.

7. Please replace the paragraph 73 with the one below:

Fig. 2 shows the localization of GFP-truncated botulinum toxin A light chain in NGF-differentiated live PC12 cells visualized on a fluorescence inverted microscope. The arrow indicates that GFP-truncated botulinum toxin A light chain localizes to punctate bodies inside the cytoplasm.

8. Please replace the paragraph 74 with the one below:

Fig. 3 shows the amino acid sequence for botulinum type A light chain. The amino acid sequence of SEQ ID NO: 29 shown, minus the underlined amino acids represents botulinum type A truncated light chain. The overline labeled $\Delta N8$ indicates the eight amino acids deleted from the amino terminus of the light chain, the overline labeled $\Delta C22$ indicates the 22 amino acids deleted from the carboxy terminus of the light chain. The double underline indicates the leucine-based motif and the dotted lines indicate tyrosine-based motifs.

9. Please replace the paragraph 75 with the one below:

Fig. 4 shows the localization of GFP-botulinum toxin A light chain with LL to AA mutation at position 427 and 428 in NGF-differentiated live PC12 cells visualized on a fluorescence inverted microscope. The arrow indicates that GFP-botulinum toxin A light chain with LL to AA mutation localizes to punctate bodies inside the cytoplasm.

10. Please replace the paragraph 76 with the one below:

Fig. 5 shows localization of fluorescently labeled anti-SNAP-25 visualized in horizontal confocal sections of staurosporine-differentiated PC12 cells. The arrow indicates that SNAP-25 localizes to the plasma membrane.

11. Please replace the paragraph 78 with the one below:

Fig. 7 shows localization of GFP-botulinum type B neurotoxin light chain in NGF-differentiated live PC12 cells visualized on a fluorescence inverted microscope. The arrow indicates that GFP-botulinum toxin B light chain localizes to punctate bodies inside the cytoplasm.

12. Please replace the paragraph 79 with the one below:

Fig. 8 shows sequence alignment and consensus sequence for botulinum toxin type A Hall A light chain of SEQ ID NO: 29 and botulinum toxin type B Danish I light chain of SEQ ID NO: 30.

13. Please replace the paragraph 81 with the one below:

Fig. 10 shows a comparison of LC/A constructs expressed from E. coli for in vitro analysis. The LC/A (WT) sequences shown are amino acids 2-14 of SEQ ID NO: 29 (Amino terminus) and amino acids 412-438 of SEQ ID NO: 29 (Carboxyl Terminus). The LC/A (Δ N8/ Δ C22) sequences shown are SEQ ID NO: 81 (Amino terminus) and SEQ ID NO: 82 (Carboxyl Terminus). The N-His LC/A (WT) sequences shown are SEQ ID NO: 83 (Amino terminus) and amino acids 412-438 of SEQ ID NO: 29 (Carboxyl Terminus).

14. Please replace the paragraph 91 with the one below:

Fig. 20 shows activity assessed by western blot of the lysate of ~~cells transfected with GFP, GFP-LCA, GFP-LCE, and GFP+LCA transfected cells.~~ Fig 20A shows the presence of the SNAP-25₁₉₇ BoNT/A cleavage product in lysates containing GFP-LCA and GFP + LCA, but not GFP alone. Fig. 20B shows the presence of the SNAP-25₁₈₀ BoNT/E cleavage product in lysates containing GFP-LCE, but not GFP alone.

15. Please replace the paragraph 92 with the one below:

Fig. 21 shows that light chain A localizes to the plasma membrane. The top panel shows that GFP alone exhibits a diffuse cytoplasmic localization. However, the bottom panel shows that GFP-botulinum toxin A light chain localizes to the plasma membrane.

16. Please replace the paragraph 93 with the one below:

Fig. 22 shows that light chain B localizes in the cytoplasm. The top panel shows that GFP-botulinum toxin B light chain exhibits a diffuse cytoplasmic localization. The bottom panel shows that botulinum toxin B light chain-GFP localizes to punctate bodies inside the cytoplasm.

17. Please replace the paragraph 94 with the one below:

Fig. 23 shows that Light Chain E also localizes primarily in the cytoplasm. The top panel shows that GFP-botulinum toxin E light chain exhibits a semi-diffuse cytoplasmic localization. The bottom panel shows that botulinum toxin B light chain-GFP exhibits a diffuse cytoplasmic localization.

18. Please replace the paragraph 98 with the one below:

Fig. 27 shows localization of Light Chains in HeLa is similar to PC12 Cells. The panel on the left shows that GFP-botulinum toxin A light chain localizes to the plasma membrane. The middle panel shows that GFP-botulinum toxin B light chain exhibits a diffuse cytoplasmic localization. The panel on the right shows that GFP-botulinum toxin E light chain exhibits a semi-diffuse cytoplasmic localization.

19. Please replace the paragraph 100 with the one below:

Fig. 29 shows HEK293T cells transfected with plasmids encoding GFP-LCA, GFP-LCE, GFP-LCB, and LCB-GFP. The panel on the left shows that GFP-botulinum toxin A light chain localizes to the plasma membrane. The middle panel shows that GFP-botulinum

toxin B light chain exhibits a diffuse cytoplasmic localization. The panel on the right shows that GFP-botulinum toxin E light chain exhibits a semi-diffuse cytoplasmic localization.

20. Please replace the paragraph 113 with the one below:

In one embodiment, the leucine-based motif is xDxxxLL (SEQ ID NO: 17), wherein x can be any amino acids. In another embodiment, the leucine-based motif is xExxxLL (SEQ ID NO: 18), wherein E is glutamic acid. In another embodiment, the duplet of amino acids can include an isoleucine or a methionine, forming xDxxxLI (SEQ ID NO: 19) or xDxxxLM (SEQ ID NO: 20), respectively. Additionally, the aspartic acid, D, can be replaced by a glutamic acid, E, to form xExxxLI (SEQ ID NO: 21), xExxxIL (SEQ ID NO: 22) and xExxxLM (SEQ ID NO: 23). In a preferred embodiment, the leucine-based motif is phenylalanine-glutamate-phenylalanine-tyrosine-lysine-leucine-leucine, ~~SEQ ID NO: 1~~ SEQ ID NO: 1.

21. Please replace the paragraph 140 with the one below:

Tyrosine-based motifs are within the scope of the present invention as biological persistence and/or a biological activity altering components. Tyrosine-based motifs comprise the sequence Y-X-X-Hy (SEQ ID NO: 24), where Y is tyrosine, X is any amino acid and Hy is a hydrophobic amino acid. Tyrosine-based motifs can act in a manner that is similar to that of leucine-based motifs. In figure 3 some of tyrosine motifs found in the type A toxin light chain are bracketed (SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, and SEQ ID NO: 38). In addition, a tyrosine-based motif is found within the leucine-based motif which is indicated by an asterisked bracket in figure 3.

22. Please replace the paragraph 143 with the one below:

Figure 8 shows a sequence alignment between type A and type B light chains isolated from strains type A HallA (~~SEQ ID NO: 19~~SEQ ID NO: 29) and type B Danish I (~~SEQ ID~~

~~NO: 20~~SEQ ID NO: 30) respectively. Light chains or heavy chains isolated from other strains of botulinum toxin types A and B can also be used for sequence comparison. The shaded amino acids represent amino acid identities, or matches, between the chains. Each of the shaded amino acids between amino acid position 10 and amino acid position 425 of the Fig. 8 consensus sequence, alone or in combination with any other shaded amino acid or amino acids, represents a biological persistence altering component that is within the scope of the present invention. For example, amino acids KAFK at positions 19 to 22 of SEQ ID NO: 29, LNK at positions 304 to 306 of SEQ ID NO: 29, L at position 228 of SEQ ID NO: 29 in combination with KL at positions 95 and 96 of SEQ ID NO: 29, FDKLYK at positions 346 to 351 of SEQ ID NO: 29, YL-T at positions 78 to 81 of SEQ ID NO: 29, YYD at positions 73 to 75 of SEQ ID NO: 29 in combination with YL at positions 78 and 79 of SEQ ID NO: 29 in combination with T at position 81 of SEQ ID NO: 29, F at position 297 of SEQ ID NO: 29 in combination with I at position 300 of SEQ ID NO: 29 in combination with KL at positions 95 and 96 of SEQ ID NO: 29 can be biological persistence altering components for use within the scope of this invention. In addition, conserved regions of charge, hydrophobicity, hydro-philicity and/or conserved secondary, tertiary, or quaternary structures that may be independent of conserved sequence are within the scope of the present invention.

23. Please replace Table 2 with the one below:

Table 2				
Toxin toxin	N-term (AAs 1-30) of LC	SEQ ID NO:	C-term (last 50 AAs) of LC	SEQ ID NO: Seq ID #
BoNT/A	MPFVNKQFNYKDPVNGVDI AYIKIPNAGQM	<u>39</u>	GFNLRNTNLAANFNGQNT INNMFNFTKLKNFTGLFEFY KLLCVRGIITSK	1440
BoNT/B	MPVTINNFNYNDPIDNDNI IMMEPPFARGT	<u>41</u>	YTIEEGFNISDKNMGKEYR GQNKAINKQAYEEISKEHL AVYKIQMCKSVK	1542
BoNT/C ₁	MPITINNFNYSDPVDNKN LYLDTHLNTLA	<u>43</u>	NIPKSNLNVLFMGQNLSRN PALRKVNPNMMLYLFTKFC HKAIDGRSLYNK	1644
BoNT/D	MTWPVKDFNYSDPVNDNDI LYLRIPQNKLI	<u>45</u>	YTIRDGFNLTKGFNIENS GQNIERNPALQKLSSSESVV DLFTKVCLRLTK	1746
BoNT/E	MPKINSFNYNDPVNDRIL YIKPGGCQEFY	<u>47</u>	GYNINNLKVNFRGQNANLN PRIITPITGRGLVKKIIRF CKNIVSVKGIRK	1848
BoNT/F	MPVAINSFNYNDPVNDDTI LYMQIPYEEKS	<u>49</u>	TVSEGFNIGNLAVNNRGQS IKLNPKIIDSIPDKGLVEK IVKFCKSVIPRK	1950
BoNT/G	MPVNIKNFNYNDPINNDDI IMMEPFNDPGP	<u>51</u>	QNEGFNIASKNLKTEFNGQ NKAVNKEAYEEISLEHLVI YRIAMCKPVMYK	2052

24. Please replace Table 3 with the one below:

Table 3				
Toxin toxin	N-term (AAs 1-30) of LC	SEQ ID NO:	C-term (last 50 AAs) of LC	SEQ ID NO: Seq ID #
BoNT/A	MPF <u>A</u> NKQFNYKDPVNGVDI AYIKIPNAGQM	<u>53</u>	GFNLRNTNLAANFNGQNT EINN <u>MN</u> RTKLKNFTGLFEFY KLLCVRGIITSK	<u>2154</u>
BoNT/A	MPFVNKQFN <u>K</u> KDPVNGVDI AYIKIPNAGQM	<u>55</u>	GFNLRNTNLAANFNGQNT EINN <u>MN</u> FTKLKN <u>AA</u> GLFEFY KLLCVRGIITSK	<u>2256</u>
BoNT/A	MPFVNKQFNYKDPVNGVDI <u>A</u> RIKIPNAGQM	<u>57</u>	GFNLRNTNLAAN <u>H</u> NGQNT EINN <u>MN</u> FTKLKNFTGLFEFY KLLCVRGIITSK	<u>2358</u>
BoNT/A	MPFVNK <u>H</u> FNYKDPVNGVDI AYIKIPNAGQM	<u>59</u>	GFNLRNTNLAANFNGQNT EINN <u>MN</u> FTKLKNFTGLFEFY KLLC <u>A</u> RGIITSK	<u>2460</u>
BoNT/B	MP <u>A</u> TINNFNYNDPIDNDNI IMMEPPFARGT	<u>61</u>	YTIEEGFNISDKNMGKEYR GQNKAINKQAYEEISKEHL AVYKI <u>R</u> MCKSVK	<u>2562</u>
BoNT/B	MPVTINNFNYNDPIDNDNI <u>I</u> <u>AA</u> EPPFARGT	<u>63</u>	YTIEEGFNISDKNMGKEYR GQNKAINKQAYEEISKEHL AV <u>R</u> KIQMCKSVK	<u>2664</u>
BoNT/B	MPVTINNFN <u>R</u> NDPIDNDNI IMMEPPFARGT	<u>65</u>	YTIEEGFNISDKNMGKEYR GQNKAINKQ <u>A</u> KEEISKEHL AVYKIQMCKSVK	<u>2766</u>
BoNT/C ₁	MPITIN <u>N</u> KNYSDPVDNKN I LYLDTHLNTLA	<u>67</u>	NIPKSNLNVLFMGQNL SRN PALRKVN PENM LYLFTKFC HK AIDGRSL <u>R</u> NK	<u>2868</u>
BoNT/D	MTWP <u>A</u> KDFNYSDP <u>A</u> NDNDI LYLRIPQNKLI	<u>69</u>	YTIRDGFNLTKGFNIENS GQNIERNPALQKL SSESVV DLFTK <u>A</u> CLRLTK	<u>2970</u>
BoNT/E	MPKINSFNYNDP <u>A</u> NDRTIL YIKPGGCQEFY	<u>71</u>	GYNINNLKVNFRGQ NANLN PRIITPITGR <u>G</u> HVKKIIRF CKNIVSVKGIRK	<u>3072</u>
BoNT/E	MPKINS <u>R</u> NYNDPVNDRTIL YIKPGGCQEFY	<u>73</u>	GYNINNLKVNFRGQ NANLN PRIITPITGRGLVKKIIRF CKN <u>AA</u> SVKGIRK	<u>3174</u>
BoNT/E	MPKINSFNYNDPVNDRTIL YIKPGGCQEF <u>R</u>	<u>75</u>	GYNINNLKVNFRGQ NANLN PRIITPITGRGLVKKIIRF CKNIVS <u>A</u> KGIRK	<u>3276</u>

BoNT/F	MP <u>A</u> AINSFNYNDPVNDDTI LYMQIPYEEKS	<u>77</u>	TVSEGFNIGNLAVNNRGQS IKLNPKIIDSIPDKGLVEK IVKFCKS <u>A</u> IPRK	33 78
BoNT/G	MPVNIKN <u>H</u> NYNDPINNDDI IMMEPFNDPGP	<u>79</u>	QNEGFNIASKNLKTEFNGQ NKAVNKEAYEEISLEHLVI YRIAMCKP <u>A</u> MYK	34 80